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Certification

On January 30, 1999, Dr. Roland Kreutzer of Weidenberg, Germany and Dr. Stefan Limmer of Bayreuth, Germany filed a patent application with the title

"Medication for Inhibiting Expression of a Defined Gene"

by submitting it to the German Patent and Trademark Office.

The attached document is a true and accurate reproduction of the original documents of this patent application.

German Patent and Trademark Office has assigned this patent application a preliminary classification of A 61 K and A 61 P according to the International Patent Classification.

Munich, May 11, 2000
German Patent and Trademark Office
President
By order
[signature]

Application No.: 199 03 713.2

Abstract

This invention relates to a medication having at least one double-stranded oligoribonucleotide (dsRNA) for inhibiting expression of a defined gene, whereby one strand of the dsRNS is complementary with this gene in at least some regions.

Medication for Inhibiting Expression of a Defined Gene

This invention relates to a medication and a use of double-stranded oligoribonucleotides.

German Patent 196 31 919 C2 describes an anti-sense RNA with special secondary structures, where the anti-sense RNA is in the form of a vector coding for it. The anti-sense RNA is an RNA molecule which is complementary to regions of the mRNA. By binding to these regions, inhibition of gene expression is achieved. This inhibition may be used in particular for diagnosis and/or treatment of diseases, e.g., tumors or viral infections. It is a disadvantage that the anti-sense RNA must be introduced into the cell in a quantity at least exactly equal to the quantity of mRNA. The efficacy of the known anti-sense methods is not especially high.

US Patent 5,712,257 describes a medication containing mismatched double-stranded RNA (dsRNA) and biologically active mismatched fragments of dsRNA in the form of a ternary complex with a surfactant. The dsRNA used here consists of synthetically produced nucleic acid single strands without a defined base sequence. The single strands enter into random base pairings, resulting in the mismatched double strands. The known dsRNA is used to inhibit the replication of retroviruses such as HIV. The genome of retroviruses consists of double-stranded RNA, which binds various proteins during the replication of the retrovirus. The bindings of these proteins and thus the replication of the virus can be inhibited if nonspecific dsRNA is introduced into the infected cells in high concentrations. This results in a competition of the nonspecific dsRNA with the double-stranded viral RNA. The inhibiting effect, i.e., the efficacy of this method is low.

It is known from A. Fire et al., Nature, vol. 391, pp. 806 that dsRNA, one of whose strands is complementary in sections with a gene of a nematode to be inhibited, will inhibit the expression of this gene with a high efficacy. It is believed that the special efficacy of the dsRNA used in cells of the nematode is not based on the anti-sense principle but instead might be attributable to catalytic properties of the dsRNA. Nothing is said in this article regarding the efficacy of specific dsRNA with respect to inhibition of gene expression, in particular in human cells.

The object of the present invention is to eliminate the disadvantages of the related art. In particular the most effective possible medication and use for production of a medication with which expression of a defined gene can be inhibited is/are to be provided.

This object is achieved by the features of Claims 1, 2, 13 and 14. Advantageous embodiments are derived from Claims 3 through 12 and 15 through 26.

According to this invention, a medication having at least one double-stranded oligoribonucleotide (dsRNA) is provided for inhibiting the expression of a defined gene, whereby one strand of the dsRNA is complementary with this gene in at least some sections. It has surprisingly been found that dsRNA is suitable as a medication for inhibiting expression of a defined gene in human cells. This inhibition is accomplished at concentrations which are at least one order of magnitude lower in comparison with the use of single-stranded oligoribonucleotides. The medication according to this invention is highly effective. Fewer side effects are to be expected.

According to another provision of this invention, a medication having at least one vector for coding double-stranded oligoribonucleotides (dsRNA) for inhibiting expression of a defined gene is provided, whereby one strand of the dsRNA is complementary to this gene in at least some sections. The proposed medication has the aforementioned advantages. Manufacturing costs in particular can be saved by the use of a vector.

If dsRNA is used as an active ingredient, it has proven advantageous that the dsRNA is packaged in micellar structures, preferably in liposomes. The dsRNA may also be enclosed in natural viral capsids or in synthetic capsids produced by chemical or enzymatic methods or structures derived therefrom. These features make it possible to introduce the dsRNA into defined target cells.

According to another feature of this embodiment, the dsRNA has 10 to 1000, preferably 250 to 350, base pairs. Such dsRNA or a vector provided for coding for same can be produced synthetically or enzymatically by conventional methods.

The gene to be inhibited, preferably an oncogene, may be expressed in eukaryotic cells or in pathogenic organisms, preferably in plasmodia. It may be a component of a virus or viroid, preferably one that is a human pathogen. The proposed medication makes it possible to treat genetically controlled diseases such as cancer and viral diseases.

The virus or viroid may also be one that is an animal or plant pathogen. In this case, the medication according to this invention also makes it possible to treat veterinary or horticultural diseases.

According to another feature of this embodiment, the dsRNA is designed to be double-stranded in some sections. Its ends may be modified to prevent degradation in the cell. This makes an enzymatic attack more difficult.

According to another provision of this invention, use of double-stranded oligoribonucleotides is provided for producing a medication for inhibiting expression of a defined gene, whereby one strand

of the dsRNA is complementary to this gene in at least some sections. Surprisingly, dsRNA is suitable for producing a medication for inhibiting expression of a defined gene. When using dsRNA, inhibition is achieved at concentrations that are lower by one order of magnitude in comparison with the use of single-stranded oligoribonucleotides. The use according to this invention thus makes it possible to produce especially effective medication.

According to another provision of this invention, use of a vector for coding for double-stranded oligoribonucleotides (dsRNA) for production of a medication for inhibiting expression of a defined gene is provided, whereby one strand of the dsRNA is complementary to this gene in at least some sections. Use of a vector makes it possible to produce especially inexpensive and effective medications.

With regard to the embodiments according to this use, reference is made to the description of the preceding features.

Exemplary embodiment:

By means of traditional methods, an RNA single strand as shown in the single accompanying sequence protocol, was synthesized enzymatically.

Furthermore, the RNA single strand complementary to it was also synthesized. Then the single strand and the complementary single strand were combined to form the dsRNA. The resulting dsRNA contains a section of the immediate early gene of the cytomegalovirus.

Experimental protocol:

A plasmid vector which could be used to synthesize the required dsRNA was constructed. For construction of this T7/SP6 transcription plasmid, a polymerase chain reaction (PCR) was performed for amplification of the 363 base pairs from the 5'-end of the immediate early gene of the cytomegalovirus. The matrix was commercially available positive control DNA from the cytomegalovirus of the HeLaScribe® nuclear extract in vitro transcription kit from Promega. The primers were oligodeoxyribonucleotides whose sequences were identical to or complementary to the ends of the range of the immediate early gene indicated above. The pGEM®-T vector (Promega) was used as the cloning vector for the resulting PCR product. Transformation of *E. coli* XI1-blue was performed. Plasmid DNA of a selected clone whose sequence had been checked by partial sequencing was linearized with NcoI or SalI and used as the matrix for in vitro transcription with SP6 or T7 RNA polymerase (RiboMAX™ in vitro transcription kit, Promega).

The resulting oligonucleotides were purified and added to sodium phosphate buffer (pH 6.5) in the presence of 100 mM NaCl in equimolar amounts. After heating to 95°C briefly, the mixture was cooled slowly over a period of approx. 2.5 hours, whereby the formation of dsRNA was achieved by pairing the two complementary single strands.

Test system with human cell nucleus extract:

Using the HeLaScribe® nuclear extract in vitro transcription kit from Promega, the transcription efficiency of the range of the immediate early gene of the cytomegalovirus indicated above was determined in the presence of the two single-stranded oligoribonucleotides and the dsRNA. This was then on the basis of the radioactivity of the [α^{32}P]ATP used as a substrate incorporated into the run-off transcripts. Separation of the free ATP from the resulting transcript was performed by gel electrophoresis. Analysis of the gel was performed with the help of a radioactivity detector (Instant Imager).

Results and conclusions:

A definite reduction in the amount of transcript in the presence of dsRNA in comparison with the control batch without RNA as well as in comparison with the batches with single-stranded RNA was found. The efficacy of the dsRNA could be achieved, even with the addition of small amounts, namely less than 10%, of the RNA concentration required for inhibition of translation in the case of the anti-sense technology. The inhibiting effect of single-stranded anti-sense RNA would not be detectable in this test system because the inhibition takes place here at the level of translation. Transcription was investigated here. The reduction in the quantity of transcript of a gene in the presence of dsRNA observed here in humans for the first time shows clearly that expression of the corresponding gene is inhibited. This effect is attributed to a novel mechanism due to the dsRNA.

Patent Claims

1. A medication having at least one double-stranded oligoribonucleotide (dsRNA) for inhibiting expression of a defined gene, one strand of the dsRNA being complementary to this gene at least in some sections.

2. The medication having at least one vector for coding double-stranded oligoribonucleotides (dsRNA) for inhibiting expression of a defined gene, whereby one strand of the dsRNA is complementary to this gene in at least some sections.

3. The medication according to Claim 1, whereby the dsRNA is packaged in micellar structures, preferably in liposomes.

4. The medication according to Claim 1, whereby the dsRNA is enclosed in natural viral capsids or in artificial capsids produced by chemical or enzymatic methods or in structures derived therefrom.

5. The medication according to one of the preceding claims, whereby dsRNA has 10 to 1000 base pairs, preferably 250 to 350 base pairs.

6. The medication according to one of the preceding claims, whereby the gene to be inhibited, preferably an oncogene, is expressable in eukaryotic cells.

7. The medication according to one of the preceding claims, whereby the gene to be inhibited is expressed in pathogenic organisms, preferably in plasmodia.

8. The medication according to one of the preceding claims, whereby the gene to be inhibited is part of a virus or a viroid.

9. The medication according to Claim 8, whereby the virus is a virus or viroid that is a human pathogen

10. The medication according to Claim 8, whereby the virus or viroid is an animal or plant pathogen.

11. The medication according to one of the preceding Claims, whereby the dsRNA is designed to be double stranded in some sections.

12. The medication according to one of the preceding Claims, whereby the ends of the dsRNA are modified to prevent degradation in the cell.

13. A use of double-stranded oligoribonucleotides (dsRNA) to produce a medication for inhibiting expression of a defined gene, whereby a strand of the dsRNA is complementary to this gene in least some sections.

14. The use of a vector for coding for double-stranded oligoribonucleotides (dsRNA) for producing a medication for inhibiting the expression of a defined gene, whereby one strand of the dsRNA is complementary to this gene in at least some sections.

15. The use according to Claim 3, whereby the dsRNA is packaged in micellar structures, preferably in liposomes.

16. The use according to Claim 13, whereby the dsRNA is enclosed in natural viral capsids or in synthetic capsids produced by chemical or enzymatic methods or in structures derived therefrom.

17. The use according to one of Claims 13 through 16, whereby the dsRNA has 10 to 1000, preferably 250 to 350 base pairs.

18. The use according to one of Claims 13 through 17, whereby the gene to be inhibited, preferably an oncogene is expressed in eukaryotic cells.

19. The use according to one of Claims 13 through 18, whereby the gene to be inhibited is expressed in pathogenic organisms, preferably in plasmodia.

20. The use according to one of Claims 13 through 19, whereby the gene to be inhibited is part of a virus or viroid.

21. The use according to Claim 20, whereby the virus is a virus or viroid that is a human pathogen.

22. The use according to Claim 20, whereby the virus or viroid is a virus or viroid that is an animal or plant pathogen.

23. The use according to one of Claims 13 through 22, whereby the dsRNA is double stranded in at least some sections.

24. The use according to one of Claims 13 through 23, whereby the ends of the dsRNA are modified to prevent degradation in the cell.

25. The use according to one of Claims 13 through 24, whereby the medication is injectable into the blood stream or into the interstitium of the organism to be treated.

26. The use according to one of Claims 13 through 25, whereby the dsRNA and/or the vector coding for it is incorporated into bacteria or microorganisms.